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Construction of *Agrobacterium* Strains by Electroporation of Genomic DNA and Its Utility in Analysis of Chromosomal Virulence Mutations

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We have extended the technique of electroporation as a genetic tool for manipulating the *Agrobacterium tumefaciens* chromosome. We used this technique to introduce chromosomal DNA into recipient *A. tumefaciens* strains by electroporation and constructed isogenic *chvE* mutants that share the same chromosomal background but differ in their types of pTi (octopine or nopaline). Both nopaline and octopine pTi-carrying *chvE* mutants were deficient in *vir* regulon induction and exhibited similar reductions in host range.

Generalized phage transduction, genomic DNA transformation, and conjugation are used in a number of bacterial systems to transfer chromosomal genes between strains (10). However, in most bacteria these methods are not available as tools for genetic study. Searching for an alternative tool that could be used for chromosomal genetic manipulation in *Agrobacterium* species, we investigated whether chromosomal genetic markers could be transferred from one strain to another by introduction of total genomic DNA by electroporation. Electroporation has been used in many biological systems as a means to introduce molecules such as DNA and proteins into cells. In many prokaryotes, including *Agrobacterium* species, electroporation has been used most extensively for the introduction of plasmid DNA, which is then maintained by autonomous replication in the recipient organism (6, 12–14, 17). We reasoned that if genomic DNA fragments could be introduced into a recipient cell, homologous recombination events between these fragments and the genomic DNA of the recipient would occur. Double crossover events would result in the replacement of the intervening DNA between the recombination sites. Such events could be selected by using appropriate genetic markers.

To test this possibility, we attempted to transfer genetic markers by electroporation. Genomic DNA was prepared from 5-ml overnight cultures as previously described (7) and was suspended in a final volume of 50 to 100 μl of TE (Tris-EDTA) buffer to give a concentration of approximately 1 μg/μl. For electroporation, 1 to 10 μl of this DNA was added to 50 μl of recipient cells that had been prepared for electroporation according to the method described by Cangelosi et al. (6), and this volume was transferred to a 2-mm electroporation cuvette on ice. Electroporation was carried out using a Bio-Rad Gene Pulser by applying a single electrical pulse of 2.5 kV with the apparatus set at 2.5 μF and 400 Ω. After receiving the pulse, the cells were suspended in 0.5 ml of MG/L (21), and the suspensions were transferred to culture tubes and incubated at 28°C with shaking. After 2 h of outgrowth, the cells were recovered by centrifugation and suspended in 0.1 ml of 0.85% NaCl or MG/L, and the suspensions were spread on appropriate selective media. Colonies arose from transformed cells after 2 to 3 days of incubation at 28°C.

It is difficult to accurately measure the efficiency of electroporation-mediated transformation, since the numbers of transformants obtained were generally very low compared with those routinely obtained with plasmid DNA. The frequency probably varies according to the quality of genomic DNA, the state of the recipient cells, and other, undefined parameters. Using 2 to 10 μg of genomic DNA, we obtained sufficient numbers of transformants to enable routine construction of strains. For example, 5 μl (~4.5 μg) of genomic DNA from the A348 *chvE*::Tn*5* strain MX1 [C58 chromosome with a *chvE*::Tn*5* allele, pTi-A6(octopine)] was electroporated into strain C58virAΔSmSp [C58 chromosome, pTi-C58(nopaline), marked by streptomycin and spectinomycin resistance] (16). Five kanamycin-resistant (from Tn*5*) transformants formed colonies, all of which retained the spectinomycin resistance marker of the recipient strain. Southern hybridization experiments, in which *Hin*dIII-cleaved genomic DNAs from the transformants were probed with labeled pGC31 DNA (carrying *chvE* on a 3.7-kb *Eco*RI fragment) (11), confirmed that the transformants carried the corresponding *chvE*::Tn*5* allele (data not shown), since they showed a hybridization pattern identical to that of the donor strain. In another experiment, the *chvE*::Tn*5* insertion in strain MX1 was introduced into the wild-type nopaline-utilizing strain C58 by electroporation, resulting in the C58 *chvE*::Tn*5* mutant At11054. Both C58 and At11054 were able to grow on AB minimal medium (7a) with 0.05% nopaline as the sole source of carbon, while MX1 could not. In addition to these examples, several other genetic markers, both chromosomal and Ti plasmid located, have successfully been transferred by this technique (data not presented). Thus, electroporation of genomic DNA should be generally applicable for genetic analysis and manipulation in organisms in which transduction and transformation of genomic DNA are not available as tools. It may be possible to increase the transformation efficiency as the method is further refined; however, this was not necessary for our purposes.

The construction of a C58 *chvE*::Tn*5* mutant, At11054, allowed us to compare the phenotypes of isogenic *chvE*::Tn*5* mutants that differ only in the type of Ti plasmid they carry, either octopine or nopaline type. *chvE* is a chromosomal gene whose product, a monosaccharide-binding protein, interacts with the Ti plasmid-encoded VirA in signal transduction (5,

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TABLE 1. Induction of *virB::lacZ* in *chvE* mutants and wild type and complementation by plasmid-carried *chvE*[a]

Strain	Ti plasmid type	*chvE* allele type	Plasmid	β-Galactosidase activity[b]	
				After incubation in the absence of AS and galactose	After incubation in the presence of AS and galactose
A348(pPTC110)	Octopine	Wild type	Vector	2.8 ± 0.3	1,159 ± 124
A348(pTC116)	Octopine	Wild type	*chvE*	2.8 ± 0.1	1,178 ± 65
MX1(pTC110)	Octopine	Mutant	Vector	3.4 ± 0.2	5.7 ± 0.4
MX1(pTC116)	Octopine	Mutant	*chvE*	3.0 ± 0.1	1,355 ± 84
C58(pTC110)	Nopaline	Wild type	Vector	14.0 ± 0.3	544 ± 46
C58(pTC116)	Nopaline	Wild type	*chvE*	4.3 ± 1.1	636 ± 16
At11054(pTC110)	Nopaline	Mutant	Vector	6.7 ± 1.0	2.5 ± 0.03
At11054(pTC116)	Nopaline	Mutant	*chvE*	7.5 ± 1.1	479 ± 8

[a] AS was added at a concentration of 5 μM, and galactose was added at a concentration of 10 mM. All strains carried the *virB::lacZ* reporter plasmid pSM243cd.
[b] The means of three assays ± standard errors of the means. Units of activity were determined as described previously (19).

11). The result is a synergistic relationship between monosaccharide and phenolic signals in *vir* gene induction. The studies which defined the role of ChvE in *Agrobacterium tumefaciens* virulence were carried out in hybrid strains consisting of octopine-type Ti plasmids in the chromosomal background of a nopaline-type strain (1, 5, 11, 18). However, in natural populations, specific types of Ti plasmids have been found to occur preferentially in certain chromosomal backgrounds (4, 15), with nopaline-type plasmids occurring in one chromosomal background and octopine type plasmids occurring in another (4). In light of the differences in virulence now apparent between octopine and nopaline strains, which are illustrated by their differing capacities for agroinfection (2, 3, 16), it became important to investigate the role of *chvE* in the virulence of nopaline strains.

The host ranges of *chvE* mutants of the nopaline chromosome-octopine pTi hybrid strain A348 are characteristically reduced, with such mutants lacking the ability to form tumors on a wide range of plants which are susceptible to infection by A348 but retaining the ability to form tumors on *Helianthus annuus* (sunflower) and *Zinnia elegans* (zinnia) (11). Virulence was examined by inoculating needle-wounded *Bryophyllum diagremontiana* (kalanchöe) leaves and stems of *Lycopersicon esculentum* (tomato), *H. annuus*, and *Z. elegans* with liquid cultures of the *Agrobacterium* strains. Tumor formation was scored after 3 weeks. Strain A136, which lacks a Ti plasmid, was used as a negative control and did not elicit tumors. The parental strains, C58 and A348, formed tumors on all plants. Strain At11054, the C58 *chvE* mutant, and the A348 *chvE* mutant MX1 exhibited similar host ranges as they were avirulent on *B. diagremontiana* and *L. esculentum* while remaining virulent on *H. annuus* and *Z. elegans*. However, the tumors formed by the *chvE* mutants on *H. annuus* were significantly smaller than the tumors formed by the wild-type strains. This may reflect reduction in the overall rate of T-DNA transfer. Dilution studies on *H. annuus* indicated that for all strains, application of 10^4 CFU per wound was sufficient to cause tumor formation after 3 weeks, whereas application of 10^2 CFU per wound was not.

To test whether full virulence could be restored to the *chvE* mutants by provision of *chvE* in *trans*, the clone pTC116 was constructed as follows. The IncW cloning vector pUCD2 (8) was cleaved with *Pvu*II and *Eco*RV and was self-ligated to yield the deletion derivative pTC110, which has a single *Eco*RI site and encodes resistance to gentamicin, kanamycin, streptomycin, and spectinomycin. The 3.7-kb *Eco*RI fragment containing *chvE* and *gbpR* (ORF1) (9, 11) was cloned into the unique *Eco*RI site of pTC110, abolishing streptomycin-spectinomycin resistance, to make pTC116. This plasmid was able to restore virulence on *B. diagremontiana* to strains MX1 and At11054.

To measure *vir* regulon induction by the phenolic inducing compound acetosyringone (AS) and the inducing monosaccharide galactose, the *virB::lacZ* fusion plasmid pSM243cd (20) was introduced into the *A. tumefaciens* strains by triparental mating from *Escherichia coli*; pTC110 and pTC116 were introduced by electroporation. The resulting strains were cultured overnight in 2 ml of MG/L broth containing carbenicillin, gentamicin, and kanamycin (each at 100 μg/ml). The cells were washed twice in glycerol induction broth (5) (pH 5.5, containing carbenicillin) and suspended in 1 ml of glycerol induction broth, and 0.1 ml of the suspension was used to inoculate 3 ml of the test solution (with or without AS and galactose), which was incubated for 16 h at 28°C with shaking. β-Galactosidase activity was measured as described previously (19). Each assay was done in triplicate.

In octopine-type Ti plasmid-carrying strains, monosaccharides such as galactose are required for full induction of the *vir* regulon, and *chvE* mutants do not respond to monosaccharides in *vir* regulon induction (5). Similarly, we found that *virB::lacZ* in strain At11054 is not induced by galactose (Table 1). Thus, ChvE is important in the regulation of *vir* genes in concert with nopaline Ti plasmid-encoded VirA. In keeping with the restoration of full virulence to At11054 by provision of *chvE* in *trans*, plasmid pTC116 also restores the ability of strain At11054 to respond to galactose in *vir* regulon induction.

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